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Parathyroid hormone linked to a collagen binding domain (PTH-CBD) promotes hair growth in a mouse model of chemotherapy-induced alopecia in a dose-dependent manner

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Abstract

Chemotherapy-induced alopecia is a major source of psychological stress in patients undergoing cancer chemotherapy, and can influence treatment decisions. While there is currently no therapy, PTH-CBD, a fusion protein of parathyroid hormone and collagen binding domain, has shown promise in animal models.

Objective—To determine if there are dose-dependent effects of PTH-CBD on chemotherapy-induced alopecia in a mouse model.

Methods—C57BL/6J mice were waxed to synchronize hair follicles; treated on day 7 with vehicle or PTH-CBD (100, 320 and 1000 mcg/kg subcutaneous injection); treated on day 9 with vehicle or cyclophosphamide (150 mg/kg i.p.). Mice were photographed every 3–4 days and sacrificed on day 63 for histological analysis. Photographs were quantified by grey scale analysis to assess hair content.

Results—Mice not receiving chemotherapy showed regrowth of hair 2 weeks following waxing, and normal histology after 2 months. Mice receiving chemotherapy alone showed marked hair loss after chemotherapy, which was sustained for 10 days and was followed by rapid regrowth of a normal coat. Histology revealed rapid cycling dystrophic anagen/catagen follicles. Animals receiving chemotherapy and PTH-CBD showed decreased hair loss and more rapid regrowth of hair than that seen with chemotherapy alone (increased hair growth by grey scale analysis, $p < 0.05$), and the effects were dose dependent. Histologically, hair follicles in animals receiving the highest dose of PTH-CBD were in a quiescent phase, similar to mice which did not receive chemotherapy.

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Conclusions—Single dose subcutaneous administration of PTH-CBD showed dose-dependent effects in minimizing hair loss and speeding recovery from chemotherapy-induced alopecia.

Keywords

Chemotherapy-induced alopecia; Cyclophosphamide; PTH-CBD; Anagen; Bone mineral density

Introduction

Chemotherapy-induced alopecia is an emotionally distressing side effect of cancer chemotherapy [1]. Most chemotherapeutic agents cause alopecia, which can be severe [2]. More than half of all people diagnosed with cancer receive chemotherapy, and approximately 65% of these develop chemotherapy-induced alopecia [3]. The alopecia side-effect can cause severe enough psychological distress to influence patient's decision making, and cause them to decide against potentially lifesaving chemotherapy regimens [4, 5]. Unfortunately, attempts to prevent or treat chemotherapy-induced alopecia have met with limited success [6, 7]. The most favorable results have been reported with a scalp cooling device [8, 9], which reduce deleterious effect of chemotherapeutic agents on the hair follicles by causing hypothermia and reducing blood flow to the scalp [10, 11]. The actual results can vary between patients and chemotherapy regimens [12], and there is a concern that scalp hypothermia may interfere with the antitumor effects of the chemotherapeutics in the scalp, increasing the risk of scalp metastasis [13–15].

PTH agonists and antagonists have been shown to modulate the hair follicle response to chemotherapy-induced damage in depilated mice, resulting in improved hair growth effects following chemotherapy [16]. To improve delivery and retention of PTH to the skin, we synthesized a fusion protein linking the agonist PTH(1–33) to a collagen binding domain (CBD) derived from ColH collagenase of *Clostridium histolyticum* [17]. We previously reported that this compound, PTH-CBD, had greater effects on hair regrowth in the depilated mouse model of chemotherapy than that seen with a similarly linked PTH antagonist [18]. We also showed beneficial effects of PTH-CBD on chemotherapy-induced alopecia in a more clinically relevant model of cyclic administration of chemotherapy in the absence of waxing [19]. We now report dose responsiveness of this effect to provide dose optimization and further verification of the efficacy of the compound.

Methods

Animals

Forty two female C57BL/6J mice aged three to five weeks old were obtained from the Jackson Laboratory, Bar Harbor, ME. They were then acclimatized for two weeks in the animal room and exposed to a 12/12 hour light/dark period at a temperature of 68–70 degrees Celsius. The mice were given access to tap water and a diet consisting of 18 percent protein purchased from Harlan Company located in Barton, IL and Madison, WI. The Institutional Animal Care and Use Committee approval for these studies was obtained from Ochsner Clinic Foundation, New Orleans, LA.

Chemicals

The chemotherapeutic agent cyclophosphamide (CYP) was obtained from the Ochsner Clinic Foundation, cancer infusion center as a solution and was used within 24 hrs. The concentration of the cyclophosphamide was 20mg/ml. The working solution of the fusion protein, PTH-CBD was prepared fresh and diluted in collagen binding buffer (CBB) (50 mM Tris Hcl, 5mM Cacl₂, pH 7.5).

Depilation

In order to provide greater uniformity of response and permit quantitative grading of the response to each dose, we utilized the depilated mouse model of chemotherapy-induced alopecia in this study. Mice were depilated by shaving followed by waxing using Sally Hansen™ (Hair Remover Wax Strip Kit – Face) strips purchased from a local vendor (Wal-Mart, Luling, LA). Depilation was done according to the manufacturers packing instructions.

Study Protocol

42 Mice were divided into five groups of 9 each and they are as follows:

1. No Chemo (6 mice)
2. Chemo (9 mice)
3. Chemo+PTH-CBD (100 mcg/kg) (9 mice)
4. Chemo+PTH-CBD (320 mcg/kg) (9 mice)
5. Chemo+PTH-CBD (1000 mcg/kg) (9 mice)

On day 0, the backs of the mice were shaved and then depilated by waxing to synchronize the hair follicles. On day 7, No Chemo and Chemo mice received a single subcutaneous injection of collagen binding buffer (CBB); the remaining groups received a single dose of either 100, 320, or 1000 mcg/kg of PTH-CBD by subcutaneous injection placed caudally in the depilated region. On day 9, 150 mg/kg of CYP was administered intraperitoneally to all animals except for the No Chemo mice. Chemotherapy administration was timed to maximize the damage to hair follicles [16]

Photo- documentation

In addition to daily observation, photo documentation was obtained every 3–4 days to provide a record of changes in hair growth. Images were captured with the KODAK Gel Logic 100 Imaging System (Eastman Kodak Company, Rochester, NY, USA), on a Spectroline^R Bi-O-Vision™ uv/white light transilluminator (Spectronics Corporation, Westbury, NY, USA). Photographs were taken with exposure 0.2 seconds, F-stop 2 mm, magnification 15 mm to keep the hair texture in the linear range for analysis. Images were evaluated qualitatively and analyzed by grey scale analysis, as previously described [19]. Briefly, light absorption within specified regions was quantified using the provided software, with greater absorption corresponding to increased hair growth. In this study, an elliptical region of interest (ROI) was selected on the dorsal skin of the mouse at the site of

injection. This densitometry value was normalized to the average of those obtained from two background ROIs placed on either side of the mouse.

Histology

Mice were sacrificed at the end of the study (day 63). Skin samples from the dorsal (middle of the back) regions were obtained. The skin was fixed in 10% buffered formalin, and longitudinal slices were processed for routine histology, using Hematoxylin and Eosin (H&E) staining.

Biochemical Assays

Serum calcium levels were measured using the QuantiChrom™ Calcium Assay Kit (DICA-500) (BioAssay Systems, Hayward, CA).

Bone mineral density measurements

Bone mineral density (BMD) was measured for the animals at 0, 2, 4, 6 and 8 weeks of the experimental period using a Hologic QDR 1000 plus DXA machine as described previously [20].

Statistical Analysis

Relative absorption from grey scale analysis was analyzed by 2-way ANOVA, followed by 1-way ANOVA at each time point and post hoc Tukey's test. Serum calcium was analyzed by ANOVA. Bone mineral density was analyzed by a 2-way ANOVA, followed by 1-way ANOVA at each time point and post hoc tests, either Bonferroni or Dunnett's as indicated for each comparison. Statistical analyses were performed using GraphPad Prism 5.0. (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Photo-documentation

No Chemo mice showed rapid regrowth of hair during the first 2 weeks after depilation on gross observation, as expected (Fig. 1). In mice treated with chemotherapy alone, there was also an initial regrowth of hair after waxing, which reversed to near-total hair loss within 1 week after receiving chemotherapy. This hair loss persisted for approximately 10 days, and was followed by rapid regrowth of a normal hair coat. In chemotherapy animals treated with PTH-CBD, there was appreciably greater regrowth of hair within the first month after chemotherapy, particularly at the site of administration and in rough proportion to the dose of PTH-CBD administered (Fig. 1). In addition, as the PTH-CBD was administered at a more caudal site, the normal cranial to caudal pattern of hair regrowth was reversed in treated animals. As would be predicted from the distribution studies [18], these effects were not restricted to the site of injection, and reduced thinning and color change in the non-depilated regions was also noticeable. In all groups, there was no observable change in hair growth in regions where the hair coat is normally thin, that is, ears and tail.

Quantitative Assessment of hair growth by grey scale analysis

Taking advantage of the black hair color in C57BL/6J mice, we performed grey scale analysis on serial photographs at the site of injection, with higher absorption corresponding to increased hair growth. Using this analysis, the No Chemo group showed marked increases in light absorption during the first 2 weeks (Fig. 2), corresponding to the time of visually apparent hair growth. As expected, this response was sustained, although there was a mild trend towards lower light absorption following day 42. Chemotherapy alone treated group showed rapid decrease in light absorption by day 18, which was sustained until day 28 (21 days after chemotherapy). There was then a marked increase in light absorption, corresponding to increased visual hair growth (Fig. 2). By day 42, light absorption did not differ between Chemo and No Chemo groups. PTH-CBD treatment appeared to have a dose-dependent effect of increased light absorption (corresponding to increased hair growth) during this window of chemotherapy-induced hair loss.

Histology

Based on our previous study, we expected animals in the No Chemo group to respond to waxing with marked stimulation of anagen VI hair follicles [18]. However, as this study was continued to a later time point, at 63 days after waxing there was no evidence of such regeneration, rather hair follicles appeared to be in a quiescent state (Fig. 3), consistent with healing after the waxing injury. Conversely, skin samples from animals treated with cyclophosphamide (Chemo group) did show marked stimulation of anagen VI hair follicles, which may indicate a delay in the healing process after waxing. We observed melanocyte clumping around the bulb of these hair follicles; a dystrophic change which has been described previously after chemotherapy administration [21]. Of note, while there were histological differences between the Chemo and No Chemo groups, the coat of the animals was visually indistinguishable.

Skin samples from the Chemo+PTH-CBD at 100 and 320 mcg/kg doses showed mixed findings, with both quiescent regions resembling those seen in No Chemo group, and regenerating regions resembling those seen in the Chemo group (Fig. 3). The skin samples from the Chemo+ PTH-CBD (1000 mcg/kg) showed only hair follicles in the quiescent phase (Fig. 3). At all doses, PTH-CBD treatment (Chemo+PTH-CBD) led to deeper rooting and reduced melanocyte clumping, thus reversing the dystrophic changes.

Bone Mineral Density

We have previously shown that PTH-CBD distributes to bone and reverses chemotherapy-induced osteoporosis following a single subcutaneous injection [17]. We therefore measured the bone mineral density in each animal at 0, 2, 4, 6 and 8 weeks of the experimental period. In the No Chemo group, we found significant increases in BMD from baseline, as would be expected in normal young mice (Fig. 4). This increase in BMD with age was delayed in the Chemo group, which showed lower BMD at 2, 4 and 6 weeks, but by 8 weeks was similar to the No Chemo group. This observation is consistent with the known effects of CYP to reduce bone formation [22, 23], although the magnitude of effect was lower than that seen with 3 courses of cyclophosphamide [17]. At the lowest dose (100 mcg/kg), PTH-CBD had no observable effect on BMD. However, at higher doses (1000 and 320 mcg/kg), PTH-CBD

increased BMD to levels at or above those seen in the No Chemo group. In fact, analysis of the BMD values at 8 weeks by ANOVA followed by Dunnett's test (comparing all groups to the Chemo group) indicated that BMD values were significantly increased after 1000 or 320 mcg/kg PTH-CBD, but not in the No Chemo group. Thus, higher doses of PTH-CBD did result in increases in BMD which corrected the deficit caused by chemotherapy administration.

Serum Calcium

As the above-mentioned changes in BMD showed evidence of systemic absorption of the CBD peptides, we measured serum calcium levels at the time of sacrifice (day 63). Serum calcium levels showed no statistically significant differences between the groups (Fig. 5).

Discussion

PTH-CBD is a fusion protein of a bacterial collagen binding domain and a PTH(1–33) agonist. This compound was designed to promote distribution and retention of the active component to collagen-containing tissues, such as skin and bone. We tested if there were dose-dependent effects of PTH-CBD on chemotherapy-induced alopecia using a depilated mouse model. Synchronizing the hair follicles by depilation (waxing) is a commonly-used model for inducing chemotherapy-induced alopecia. It has the advantage of maximizing chemotherapy-induced damage with a single course of chemotherapy, minimizing other systemic effects of the chemotherapeutics and improving survival. Importantly for this study, it provides a more uniform platform for assessing drug responses, in that the response of the animals is more consistent. Unfortunately, waxing itself causes injury to hair follicles, for which there is a persistent anagen response at 30 days in mice which do not receive chemotherapy [18]. We therefore extended this study for another month, and observed that this anagen response resolves by day 63, with normal quiescent hair follicles observed on histology. Thus, this extended model provides the opportunity to assess if therapeutic agents can return the hair follicles to a truly normal state.

Taking advantage of the uniformity of this model, we compared hair growth responses visually, quantitatively by grey scale analysis, and histologically by assessing the stage and dystrophy of hair follicles. We observed that PTH-CBD, had positive, dose-dependent effects in all these areas, speeding regrowth of hair and resolving chemotherapy-induced hair follicle dystrophy, [18]. These findings were confirmed by grey scale analysis, which showed that the animals treated with chemotherapy and PTH-CBD exhibited significantly greater average light absorption on the dorsum compared to those receiving chemotherapy alone, again in a dose-dependent manner.

While mice treated with CYP alone showed normal hair coat at the end of the study (day 63), the histological appearance was quite different from animals which did not receive chemotherapy. The hair follicles were still regenerating and were predominantly in the dystrophic anagen and catagen phases, while samples from the No Chemo group showed normal telogen hair follicles (Fig. 3). Animals treated with the highest dose of PTH-CBD also showed normal hair coat; however, histology showed the hair follicles to be in a quiescent phase, similar to animals that did not receive chemotherapy. Animals receiving a

lower dose of PTH-CBD showed a mixed response on histological examination, with both regenerating and quiescent regions present on the same skin sample. These observations suggest that PTH-CBD treatment promoted a full recovery from both waxing injury and chemotherapy injury.

This accelerated healing response likely resulted from an early stimulation of hair follicle regeneration by PTH-CBD; indeed, we did observe accelerated anagen responses to PTH-CBD at the 30 day time point in a previous study [18]. Activation of parathyroid hormone receptors has been shown to increase production of beta-catenin [24], which in turn induces hair follicle transition to the anagen phase [25, 26]. While in this phase, the hair follicles may be more susceptible to damage from chemotherapy; however, the continued regenerative stimulus provided by PTH-CBD would result in more rapid repair of this damage, with a net positive effect on hair growth after chemotherapy.

The lack of continued anagen response 63 days after PTH-CBD dosing might suggest that the medication effects have worn off by this time point. This is different from more prolonged anabolic effects seen in bone 9–12 months after a single subcutaneous injection of PTH-CBD [17], and may be the result of increased metabolic activity and increased blood flow to skin vs. bone [17]. Indeed, animals in this study treated with Chemo+PTH-CBD (320 or 1000 mcg/kg) showed higher BMD than that seen in Chemo mice, and by 8 weeks the BMD was higher than that seen in all other groups, including the No Chemo mice. Thus, while skin effects of PTH-CBD may have been waning by day 63, there was evidence of a continuing anabolic bone effect. Of note, despite this evidence of systemic absorption of PTH-CBD, there was no difference in serum calcium between groups at the end of the study (Fig. 5). It is possible that these hair growth effects at the site of administration may be prolonged and systemic absorption minimized using variations of PTH-CBD with enhanced collagen binding activity.

Overall, PTH-CBD showed dose-dependent effects in minimizing and speeding recovery from chemotherapy-induced alopecia. This was evident based on gross observation, quantification of hair growth using a novel grey scale analysis technique, and on histological examination. PTH-CBD also reversed effects of chemotherapy-induced bone loss, indicating that there was significant systemic absorption. Future studies will investigate if systemic effects can be minimized through topical administration and/or use of variations of PTH-CBD with enhanced collagen binding activity. Ultimately such compounds may help provide relief for cancer patients from chemotherapy-induced alopecia.

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References

1. Nakashima-Kamimura N, Nishimaki K, Mori T, Asoh S, Ohta S. Prevention of chemotherapy-induced alopecia by the anti-death FNK protein. *Life Sci.* 2008; 82:218–225. [PubMed: 18164732]
2. D'Agostini F, Fiallo P, Ghio M, De Flora S. Chemoprevention of doxorubicin-induced alopecia in mice by dietary administration of L-cystine and vitamin B6. *Arch Dermatol Res.* 2013; 305:25–34. [PubMed: 22699427]
3. Wikramanayake TC, Amini S, Simon J, Mauro LM, Elgart G, Schachner LA, et al. A novel rat model for chemotherapy-induced alopecia. *Clin Exp Dermatol.* 2012; 37:284–289. [PubMed: 22409523]
4. McGarvey EL, Baum LD, Pinkerton RC, Rogers LM. Psychological sequelae and alopecia among women with cancer. *Cancer Pract.* 2001; 9:283–289. [PubMed: 11879330]
5. Trueb RM. Chemotherapy-induced hair loss. *Skin Therapy Lett.* 2010; 15:5–7. [PubMed: 20700552]
6. Cece R, Cazzaniga S, Morelli D, Sfondrini L, Bignotto M, Menard S, et al. Apoptosis of hair follicle cells during doxorubicin-induced alopecia in rats. *Lab Invest.* 1996; 75:601–609. [PubMed: 8874390]
7. Jimenez JJ, Roberts SM, Mejia J, Mauro LM, Munson JW, Elgart GW, et al. Prevention of chemotherapy-induced alopecia in rodent models. *Cell Stress Chaperones.* 2008; 13:31–38. [PubMed: 18347939]
8. Christodoulou C, Tsakalos G, Galani E, Skarlos DV. Scalp metastases and scalp cooling for chemotherapy-induced alopecia prevention. *Ann Oncol.* 2006; 17:350. [PubMed: 16166175]
9. Ron IG, Kalmus Y, Kalmus Z, Inbar M, Chaitchik S. Scalp cooling in the prevention of alopecia in patients receiving depilating chemotherapy. *Support Care Cancer.* 1997; 5:136–138. [PubMed: 9069614]
10. Cline BW. Prevention of chemotherapy-induced alopecia: a review of the literature. *Cancer Nurs.* 1984; 7:221–228. [PubMed: 6372990]
11. Grevelman EG, Breed WP. Prevention of chemotherapy-induced hair loss by scalp cooling. *Ann Oncol.* 2005; 16:352–358. [PubMed: 15642703]
12. Komen MM, Smorenburg CH, van den Hurk CJ, Nortier JW. Factors influencing the effectiveness of scalp cooling in the prevention of chemotherapy-induced alopecia. *Oncologist.* 2011; 18:885–891. [PubMed: 23650021]
13. Balsari AL, Morelli D, Menard S, Veronesi U, Colnaghi MI. Protection against doxorubicin-induced alopecia in rats by liposome-entrapped monoclonal antibodies. *Faseb J.* 1994; 8:226–230. [PubMed: 8119493]
14. Poder TG, He J, Lemieux R. [Effectiveness of scalp cooling in chemotherapy]. *Bull Cancer.* 2011; 98:1119–1129. [PubMed: 21914579]
15. Lemieux J, Desbiens C, Hogue JC. Breast cancer scalp metastasis as first metastatic site after scalp cooling: two cases of occurrence after 7- and 9-year follow-up. *Breast Cancer Res Treat.* 2011; 128:563–566. [PubMed: 21431870]
16. Peters EM, Foitzik K, Paus R, Ray S, Holick MF. A new strategy for modulating chemotherapy-induced alopecia, using PTH/PTHrP receptor agonist and antagonist. *J Invest Dermatol.* 2001; 117:173–178. [PubMed: 11511291]
17. Ponnappakkam T, Katikaneni R, Suda H, Miyata S, Matsushita O, Sakon J, et al. A single injection of the anabolic bone agent, parathyroid hormone-collagen binding domain (PTH-CBD), results in sustained increases in bone mineral density for up to 12 months in normal female mice. *Calcif Tissue Int.* 2012; 91:196–203. [PubMed: 22806683]
18. Katikaneni R, Ponnappakkam T, Suda H, Miyata S, Sakon J, Matsushita O, et al. Treatment for chemotherapy-induced alopecia in mice using parathyroid hormone agonists and antagonists linked to a collagen binding domain. *Int J Cancer.* 2012; 131:E813–E821. [PubMed: 22130912]
19. Katikaneni R, Ponnappakkam T, Matsushita O, Sakon J, Gensure R. Treatment and prevention of chemotherapy-induced alopecia with PTH-CBD, a collagen-targeted parathyroid hormone analog, in a non-depilated mouse model. *Anticancer Drugs.* 2013; 2013:9.

20. Katikaneni R, Ponnappakkam A, Miller E, Ponnappakkam T, Gensure RC. A new technique for precisely and accurately measuring lumbar spine bone mineral density in mice using clinical dual energy X-ray absorptiometry (DXA). *Toxicol Mech Methods*. 2009; 19:225–231. [PubMed: 19750018]
21. Hendrix S, Handjiski B, Peters EM, Paus R. A guide to assessing damage response pathways of the hair follicle: lessons from cyclophosphamide-induced alopecia in mice. *J Invest Dermatol*. 2005; 125:42–51. [PubMed: 15982301]
22. Kim SH, Lim SK, Hahn JS. Effect of pamidronate on new vertebral fractures and bone mineral density in patients with malignant lymphoma receiving chemotherapy. *Am J Med*. 2004; 116:524–528. [PubMed: 15063813]
23. Hirbe A, Morgan EA, Uluckan O, Weilbaecher K. Skeletal complications of breast cancer therapies. *Clin Cancer Res*. 2006; 12:6309s–6314s. [PubMed: 17062720]
24. Dunbar ME, Dann P, Brown CW, Van Houton J, Dreyer B, Philbrick WP, et al. Temporally regulated overexpression of parathyroid hormone-related protein in the mammary gland reveals distinct fetal and pubertal phenotypes. *J Endocrinol*. 2001; 171:403–416. [PubMed: 11739006]
25. Huelsken J, Vogel R, Erdmann B, Cotsarelis G, Birchmeier W. beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell*. 2001; 105:533–545. [PubMed: 11371349]
26. Lei MX, Chuong CM, Widelitz RB. Tuning Wnt signals for more or fewer hairs. *J Invest Dermatol*. 2013; 133:7–9. [PubMed: 23299447]

Figure 1. Photo-documentation – Day 24

Seven week-old female C57BL/6J mice received single subcutaneous injections of either vehicle control or of PTH-CBD at the indicated doses on day 7, followed by treatment with cyclophosphamide (intraperitoneally) or vehicle control (No Chemo group) on day 9. Shown here are photographs of mice obtained at day 24. Arrow indicates site of injection.

A)

B)

Figure 2. Grey Scale Analysis

Images were captured using a Kodak Gel Logic 100 Imaging System on a Spectroline Bi-O-Vision UV/white light transilluminator. An elliptical region of interest (ROI) was selected on the dorsal skin of the mice, covering the region around the site of injection (see Fig. 1). Absorption in this ROI was normalized to the average absorption of two ROIs placed on either side of the mouse. A) Relative absorption from grey scale analysis, analyzed by 2-way ANOVA followed by 1-way ANOVA at each time point and post-hoc testing by Tukey's test. B) Relative absorption from grey scale analysis on day 24, which showed the greatest

different between the treatment doses, analyzed by 1-way ANOVA followed by Tukey's test.

* = $P < 0.05$ vs chemo alone. # = $P < 0.05$ vs Chemo+PTH-CBD (100 mcg/kg).

No Chemo

Chemo

Chemo+PTH-CBD (100 mcg/kg)

Chemo+PTH-CBD (1000 mcg/kg)

Figure 3. Histological Analysis (4x)

Seven week-old female C57BL/6J mice received single subcutaneous injections of either vehicle control or of PTH-CBD at the indicated doses on day 7, followed by treatment with cyclophosphamide (intraperitoneally) or vehicle control (No Chemo group) on day 9. Mice were sacrificed at day 63. Skin samples were obtained from the back at the site of peptide injection. The skin was fixed in 10% buffered formalin, and longitudinal slices were processed for routine histology, using hematoxylin and eosin (H&E) staining.

Figure 4. Bone Mineral Density (BMD)

Seven week-old female C57BL/6J mice received single subcutaneous injections of either vehicle control or of PTH-CBD at the indicated doses on day 7, followed by treatment with cyclophosphamide (intraperitoneally) or vehicle control (No Chemo group) on day 9. Animals were anesthetized, and the lumbar spine BMD was measured by DXA using a Hologic QDR-1000plus at 0, 2, 4, 6 and 8 weeks. The analysis was performed by the same investigator in a blinded fashion. Results are expressed as mean $\pm$ standard deviation. BMD was analyzed by a 2-way ANOVA, followed by 1-way ANOVA at each time point and post hoc tests, either Bonferroni or Dunnett's as indicated for each comparison.

*= $p < 0.05$ by 1-way ANOVA.

Figure 5. Serum Calcium

Seven week-old female C57BL/6J mice received single subcutaneous injections of either vehicle control or of PTH-CBD at the indicated doses on day 7, followed by treatment with cyclophosphamide (intraperitoneally) or vehicle control (No Chemo group) on day 9. Blood samples were obtained at the end of the study (day 63) and serum calcium was measured using the QuantiChrom™ Calcium Kit (Bioassay Systems, Hayward, CA). Results are expressed as mg/dl, mean $\pm$ standard deviation. Serum calcium was analyzed by ANOVA.